The contribution of phenolic acids to the anti-inflammatory activity of mushrooms: screening in phenolic extracts, individual parent molecules and synthesized glucuronated and methylated derivatives

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LIST OF ABREVIATIONS

5-LOX	5-Lipooxygenase
ANOVA	One-way analysis of variance
CA	Cinnamic acid
CAGP	Cinnamic acid glucuronide protected form
CAM	Methyl 3-phenylacrylate
cGMP	Current Good Manufacturing Practice
CoA	*p*-Coumaric acid
CoAGP	*p*-Coumaric acid glucuronide protected form
CoAM1	Methyl 3-(4-hydroxyphenyl) acrylate
CoAM2	Methyl 3-(4-methoxyphenyl) acrylate
CoAM3	3-(4-Methoxyphenyl) acrylate
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
Da	Dalton
DCP	*p*-Dicoumaroylputrescine
DFP	Diferuloylputrescine
DMEM	Dulbecco's modified Eagle's minimum essential medium
DMSO	Dimethyl sulfoxide
EC_{50}	Extract concentration providing 50% of inhibition of the NO production
FA	Ferulic acid
FBS	Fetal bovine serum
HA	*p*-Hydroxybenzoic acid
HADs	Hydroxycinnamic acid derivatives
HAGP	*p*-Hydroxybenzoic acid protected form
HAM1	Methyl 4-hydroxybenzoate
HAM2	methyl-*p*-anisate
HAM3	4-Methoxybenzoic acid
HPLC-PDA	High performance liquid chromatography with a photodiode array detector
ICAM-1	Intercellular adhesion molecule-1
IL-1β	Interleukin 1β
IL-6	Interleukin 6

IL-8	Interleukin 8
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
NED	N-(1-napthyl) ethylenediamine hydrochloride
NF-Kb	Nuclear factor-κB
NO	Nitric oxide
NSAIDs	Nonsteroidal anti-inflammatory drugs
OH	Hydroxyl
PGE2	Prostaglandin E2
PDA	Photodiode array detector
SD	Standard deviation
SRB	Sulphorodamine B
TNF-α	Tumour necrosis factor α)
UV	Ultraviolet
UFLC	Ultra-fast liquid chromatograph

ABSTRACT

In the present study, the ethanolic extracts of fourteen wild edible mushrooms were investigated for their anti-inflammatory potential in LPS (Lipopolysaccharide) activated RAW 264.7 macrophages. Furthermore the extracts were chemically characterized in terms of phenolic acids and related compounds. The identified parent molecules (*p*-hydroxybenzoic, *p*-coumaric and cinnamic acids) and their synthesised glucuronated and methylated derivatives obtained by chemical synthesis were evaluated for the same bioactivity, in order to establish a structure-activity relationship, and to understand the contribution of the compounds to the activity of the extracts. The extract *Pleurotus ostreatus, Macrolepiota procera, Boletus impolitus* and *Agaricus bisporus* revealed the strongest anti-inflammatory potential, presenting also the highest concentration in cinnamic acid, which was also the individual compound with the highest anti-inflammatory activity. The derivatives of *p*-coumaric acid revealed the strongest properties, especially the compound CoA-M1, that exhibited a very similar activity to the one showed by dexamethasone used as anti-inflammatory standard; by contrast, the *p*-hydroxybenzoic derivatives revealed the lowest inhibition of NO production. All in all, whereas the conjugation reactions change the chemical structure of phenolic acids and may increase or decrease their activity, the glucuronated and methylated derivatives of the studied compounds are still displaying anti-inflammatory activity.

CHAPTER 1. INTRODUCTION

1.1. Inflammation and conventional anti- inflammatory drugs

Inflammation is considered to be part of the complex biological response to remove injury or harmful stimuli such as pathogens, damaged cells, or irritation and this is a central feature of many pathophysiological conditions such as atherosclerosis, obesity, metabolic syndrome, diabetes (Pradhan, 2007) and even several types of cancers (Moro et al., 2012).

When cells are exposed to immune stimulants, the pro-inflammatory cells, such as macrophages, monocytes, or other host cells, start to produce cytokines and other mediators, which initiate the inflammation process. Among the various inflammatory mediators, the most common are interleukins (IL-1β, IL-6, IL-8), tumour necrosis factor (TNF-α), nuclear factor-κB (NF-κB), intercellular adhesion molecule-1 (ICAM-1), inducible type cyclooxygenase- (COX-2), prostaglandin E2 (PGE2), 5-lipooxygenase (5-LOX), and inducible nitric oxide synthase (iNOS), which leads to the production of reactive nitrogen species such as nitric oxide (NO). Overproduction of these inflammatory mediators lead to different kinds of cell damage (Kanwar et al., 2009).

Macrophages are large specialized cells that engulf and digest cellular debris, microbes, and cancer cells in a process called phagocytosis. More than a century has passed since Metchnikoff in 1892 first termed large phagocytotic cells ``macrophages`` and described their presence in the spleen, liver and connective tissues of vertebrates where they display anatomical and functional diversity (Takahashi, 2000). They play important role in non-specific host defence mechanism and help to initiate other defence mechanisms. Beyond stimulating the immune system, macrophages play a crucial role in the inflammatory response through the release of a variety of factors, such as NO, prostaglandin mediators and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), in response to an activating stimulus, e.g. lipopolysaccharide (Moro et al., 2012).

The class of anti-inflammatory drugs (steroidal and non-steroidal) are among the most widely prescribed groups of medicines in clinical practice worldwide. Recently, the nonsteroidal anti-inflammatory drugs (NSAIDs) are usually the most commonly administrated drugs to

reduce inflammation in the body. Many studies, however, have shown that the long-term administration of NSAIDs has the potential for significant side effects on the gastrointestinal tract with numerous harmful effects such as mucosal lesions, bleeding, peptic ulcers, and intestinal perforation (Dugowson et al., 2006). Recent studies show that NSAIDs are also associated with a relatively high incidence of renal adverse drug reactions, nephrotic syndrome, high blood pressure, acute tubular necrosis and cardiovascular toxicity (Elsayed et al., 2014). In this context, the discovery of new prototypes with improved anti-inflammatory activity and therapeutic safety is one of the targets in the area of research, development and innovation of the pharmaceutical industry.

The anti-inflammatory action of NSAIDs can be explained by the effect of inhibiting the enzyme COX, which is responsible for the synthesis of prostaglandins, mediators with a great ability to induce inflammation (Tanaka et al., 2009). COX has two isoforms: COX-1 and COX-2. The first is constitutively expressed in a wide variety of cells, promoting physiological functions, such as gastric mucosal protection, control of renal blood flow, hemostasis, autoimmune responses, lungs, central nervous system, cardiovascular system and reproductive functions (Grosser et al., 2006). On the other hand, COX-2 is an inductive enzyme, which is expressed significantly due to various stimuli such as cytokines, endotoxins and growth factors. COX-2 originates inducing prostaglandins, which contribute to the development of the four cardinal signs of inflammation: pain, heat, redness and swelling (Fitzgerald, 2004), thus being considered as the main target for the anti-inflammatory action. In this context, the recently developed coxibs act through selective inhibition of COX-2. However, although inductive, this COX isoform is also expressed in normal vascular endothelial cells, synthesizing prostacyclin, an important substance in maintaining the prothrombotic/antithrombotic blood balance (Antman et al., 2007), which can trigger severe cardio-vascular problems.

1.2. Mushrooms as a source of alternative anti-inflammatory compounds

Mushrooms are macrofungi with distinctive and visible fruiting bodies (**Figure 1**) that are either epigeous with fruiting bodies above the ground or hypogeous with underground fruiting bodies (Hesham et al., 2013).

Mushrooms have been widely appreciated all over the world not only for their nutritional properties (Kalac, 2009), but also for their pharmacological value related with their richness in many bioactive metabolites of high medicinal value such as lectins, polysaccharides, phenolic compounds, terpenoids, ergosterol and derivatives, and volatile organic compounds (Hesham et al., 2013). They have been described as sources of important bioactive compounds such as antioxidants (Puttaraju et al., 2006; Ferreira et al., 2009, Heleno et al., 2015), antitumors (Moradali et al., 2007; Ferreira et al., 2010, Carocho et al 2013), antimicrobials (Alves et al., 2012; Alves et al., 2013), immunomodulators (Borchers et al., 2008), antiatherogenics (Mori et al., 2008) and hypoglycemic (Hu et al., 2006) compounds. Due to these properties, they have been recognized as functional foods, and as a valuable source of natural medicines and nutraceuticals (Lindequist et al., 2005; Guillamón et al., 2010; Heleno et al., 2013).

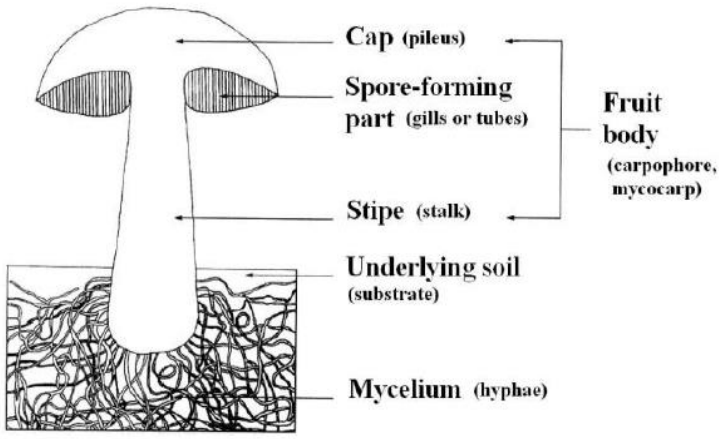


Figure 1. Structure of a mushroom with mycological terms used (Kalac, 2009).

Moreover, mushrooms have also demonstrated some anti-inflammatory potential based on their ability to reduce the production of inflammatory mediators (Padilha et al., 2009; Elsayed

et al., 2014). Previous research studies have been carried out on this regarding with several mushroom species. For example, water, methanolic, ethanolic, and ethyl acetate extracts of different mushrooms showed significant decreases in the activities of inflammatory mediators such as NO, cytokines, and prostaglandins, thus inhibiting some macrophage functions and reducing cell inflammations (**Table 1**). Different compounds have been pointed out as the responsible for the anti-inflammatory activity such as β-glucans (Nosálóva et al., 2001), triterpenes (Ma et al., 2013, Choi et al., 2014), glycoproteins (Gunawardena et al., 2014) and even phenolic compounds (Moro et al., 2012). However, not too much is known about the phenolic bioactive forms *in vivo*; these compounds are metabolized and circulate in the organism as glucuronated, sulfated and methylated metabolites, displaying higher or lower bioactivity (Heleno et al., 2015).

Table 1. Some previous studies on anti-inflammatory activity of different mushroom species evaluated through NO assay.

Species	Country	Extract	Inhibition of NO production	References
Agaricus bisporus	Spain	Methanol	30% at 0.5 mg/mL	Moro et al., 2012
	Australia	Ethanol	50% at 0.032 mg/mL	Gunawardena et al., 2014
Boletus edulis	Spain	Methanol	10% at 0.5 mg/mL	Moro et al., 2012
Cantharellus cibarius	Spain	Methanol	70% at 0.5 mg/mL	Moro et al., 2012
Caripia montagnei	Brazil	Acetone 80%, methanol	43% at 10 mg/kg 54% at 30 mg/kg 49% at 50 mg/kg	Queiroz et al., 2010
Craterellus cornucopoides	Spain	Methanol	55% at 0.5 mg/mL	Moro et al., 2012
Flammulina velutipes	Australia	Ethanol	50% at 0.024 mg/mL	Gunawardena et al., 2014
Inonotus obliquus	China	Ethanol	65% at 40 µg/mL	Ma et al., 2013
	South Korea	Ethanol	50% at 89 µg/mL	Park et al., 2005
Lactarius deliciosus	Spain	Methanol	40% at 0.5 mg/mL	Moro et al., 2012
Lentinus edodes	Australia	Ethanol	50% at 0.027 mg/mL	Gunawardena et al., 2014
Pleurotus ostreatus	Australia	Ethanol	50% at 0.077 mg/mL	Gunawardena et al., 2014
	Spain	Methanol	15% at 0.5 mg/mL	Moro et al., 2012
Pleurotus tuber-regium	Belgium	Ethanol	70% at 0.5 mg/mL	Liu et al., 2014
Tricholoma matsutake	South Korea	Dichloromethane	47% at 2 mg/mL	Lim et al., 2007

1.2.1. Carbohydrates

Carbohydrates have been known to possess some interesting biological activity in terms of prevention and treatment of common diseases (Villares, 2013). Mushrooms have been known as a valuable source of bioactive carbohydrates hence, several polysaccharides and bioactive carbohydrates have been extracted from these species (Villares, 2013).

Polysaccharides can be considered as one of the main group of carbohydrate compounds occurring in mushrooms that are responsible for a great range of healthy properties. They are the major components in mushrooms and the total content ranges from 35% to 70% in dry weight. Most of the carbohydrates in mushrooms are non-digestible carbohydrates (dietary fiber) including oligosaccharides (e.g., trehalose) and cell wall polysaccharides (e.g. chitin, β-glucans (**Figure 2**) and mannans) (Cheung, 2010; 2013).

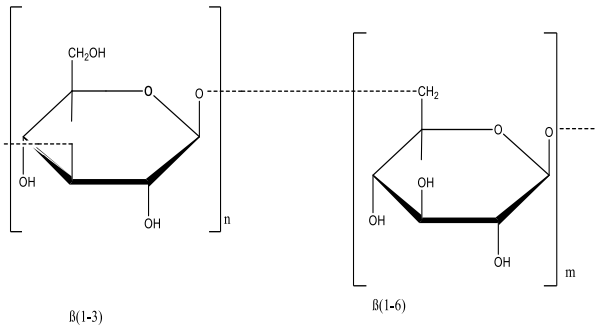


Figure 2. Basic chemical structure of β-glucans**.**

Several studies have shown the potential of polysaccharides as anti-inflammatory agents; the methanolic extract of *Caripia montagnei* (Berk.) Kuntze, was studied and the results suggest that its glucans are effective immunomodulators with anti-inflammatory potential (Queiroz et al., 2010).

Furthermore, the aqueous extract of fuco-galactan from *Agaricus bisporus* (J.E. Lange) Emil J. Imbach, exhibited anti-inflammatory response in male Swiss mice (Elsayed et al., 2014). Different structural patterns have been found within the polysaccharides extracted from mushrooms. In this field, it is generally accepted that the β(1→3),(1→6)-linked carbohydrates play an important role in enhancing the antitumor and the immunomodulatory effects.

1.2.2. Terpenes

Terpenes are a large and diversified group of organic compounds occurring widely in plants, macrofungi and animals, and empirically regarded as built up from isoprene, a hydrocarbon consisting of five carbon atoms with molecular formula $(C_5H_6)n$ as building blocks (Hesham et al., 2013). The terpene compounds are named based on the number of repeated units of terpene building blocks, for example, monoterpenes (10 carbons), sesquiterpenes (15 carbons), diterpenes (20 carbons), sesterterpenes (25 carbons), triterpenes (30 carbons), and tetraterpenes (40 carbons). These compounds exist widely in plants as the main components of resin and essential oil. In macrofungi, terpenes are present in modified forms (terpenoids or isoprenoids) and show biological activities with potential medical applications **(**Hesham et al., 2013).

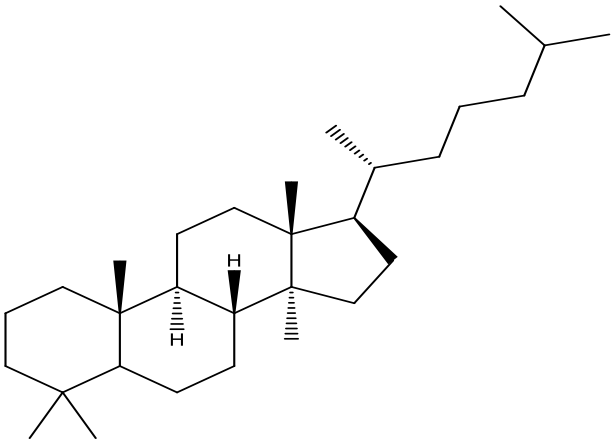


Figure 3. Chemical structure of lanostane.

Mushrooms belonging to *Ganoderma* sp., such as *Ganoderma lucidum* (Curtis) P. Karst., and *Ganoderma applanatum* (Pers.) Pat., are known for their high content of triterpenoids, for example, lanostane (**Figure 3**), which shows immumomodulating and anti-infective activities (Jeong et al., 2008). Choi et al. (2014) investigated the anti-inflammatory effects of 12 triterpenes from *G. lucidum*, determining whether these triterpenes inhibit LPS induced NO production as a marker for inflammatory responses in RAW264.7 cells. (Dudhgaonkar et al., 2009). The authors evaluated the anti-inflammatory effects of the triterpene extract from *G. lucidum* in LPS-stimulated macrophages and the results were found to be interesting.

Recent research has shown that some *G. lucidum* terpenes can prevent drug nephrotoxicity and inflammation, suggesting that they may have pharmacological applications (Elsayed et al., 2014).

1.2.3. Peptides

Peptides have been the target of intense research and development in recent years by the pharmaceutical and biotechnology industry. Bioactive proteins and peptides were reported from many sources ranging from bacteria, plants, macrofungi to humans. Many of them show bioactive characteristics like antibacterial, antifungal, antiparasitic, antihypertensive, antisporadical, and anticancer activities, among many others.

In addition to the previous bioactive compounds, anti-inflammatory peptides of different molecular weights have been isolated from mushrooms. A low molecular weight peptide known as Cordymin with molecular weight of 10,906 Da, has been identified and purified from the medicinal mushrooms *Cordyceps sinensis* (Berk.) G. H. Sung, J. M. Sung, Hywel-Jones and Spatafora, and *Cordyceps militaris* (L.) Fr. (Wang et al., 2012). This peptide significantly inhibited the infiltration of polymorphonuclear cells and IR-induced up-regulation of C_3 protein produced in the brain, interleukin-1β, and TNF-α, which had a neuroprotective effect on the ischemic brain, due to the inhibition of inflammation. Also, another important anti-inflammatory peptide is Agrocybin that has been isolated from the edible mushroom *Agrocybe cylindracea* (V. Brig.) Singer (Elsayed et al., 2014).

1.2.4. Phenolic Compounds

Mushrooms have been known to produce an amazing diversity of secondary metabolites. One of the most important groups of these metabolites are phenolic compounds. Phenolics are characterized by at least one aromatic ring (C6) bearing one or more hydroxyl groups **(**Rice-Evans et al., 1997). Phenolic compounds, including phenolic acids, are a group of secondary metabolites from fungi and plants secreted for protection against UV light, insects, viruses and bacteria (Heleno et al., 2015).

The term phenolics encompasses approximately 8000 naturally occurring compounds and current classification divides the broad category of phenols into several different groups, distinguished by the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton (simple phenols, benzoic acids, phenylopropanoids and flavonoids; Michalak, 2006). Phenolic compounds have received considerable attention for being potentially protective factors against cancer and heart diseases, in part because of their

potent antioxidative properties and their ubiquity in a wide range of commonly consumed foods (Jahangir et al., 2009). Furthermore, Kim et al. (2012) described corn bran (80% ethanolic extract) as a source of anti-inflammatory agents such as four hydroxycinnamic acid derivatives, including two free cinnamic acids, *p*-coumaric acid and ferulic acid, and their conjugate phenolic amides, *p*-dicoumaroylputrescine and diferuloylputrescine.

1.3. The particular case of phenolic acids

Phenolic acids are synthesized from the shikimate pathway from L-phenylalanine or L-tyrosine (Rice-Evans et al., 1996) (**Figure 4**). Phenylalanine and tyrosine are very important amino acids in this pathway since these amino acids are the common precursors for the majority of the natural phenolic products (Valentão et al., 2005; Baptista et al., 2007).

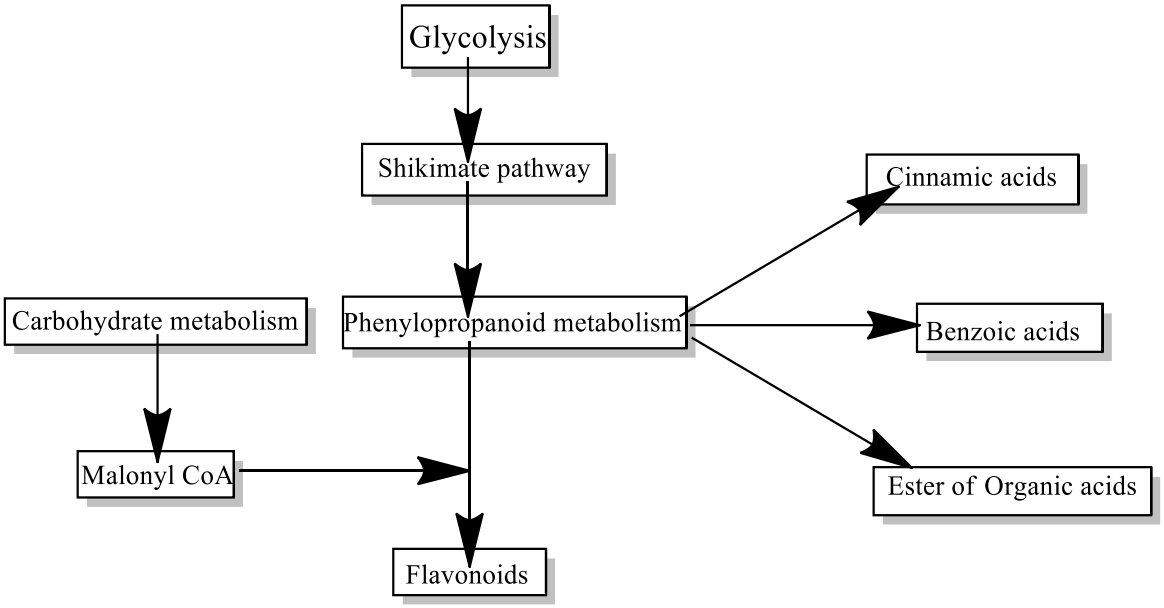
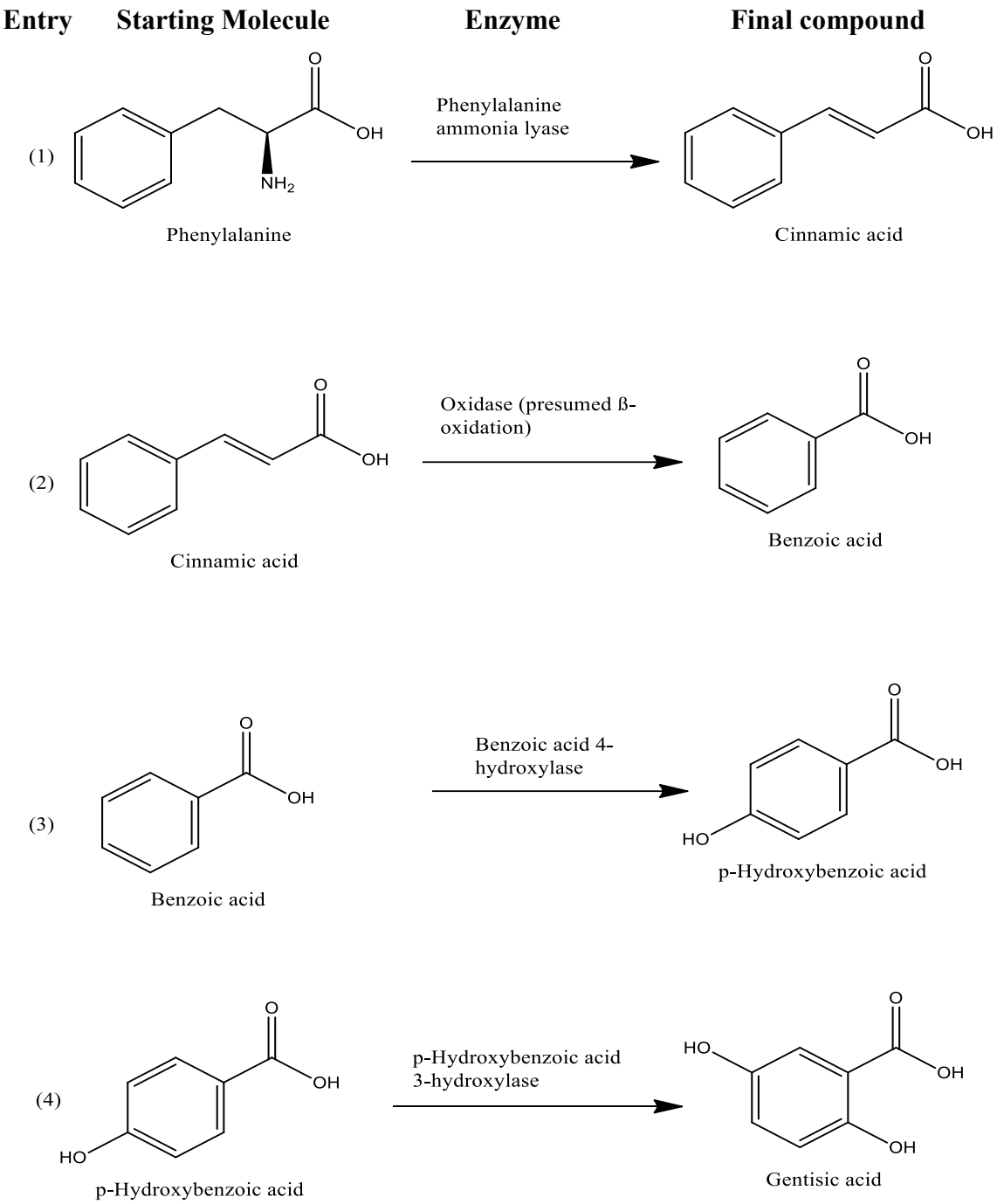


Figure 4. Biosynthesis pathways leading to formation of main groups of phenolic compounds (Ryan et al., 1999).

In the past few decades, mushrooms have received special attention for being rich sources of phenolic acids that are amongst the major contributors to their medicinal properties (Ferreira et al., 2009). Extensive knowledge of phenolic acids bioavailability is essential to understand the conjugations and bioactivities in the organism. Phenolic acids are a considerable group inside the polyphenol family, and there is evidence that when they are absorbed in the free form (as are mostly found in mushrooms), their absorption and conjugation (specially

glucuronation), follows the same pathways as that of flavonoids and other polyphenols (Cremin et al., 2001).

Phenolic acids can be divided into two major groups, hydroxybenzoic acids and hydroxycinnamic acids, which are derived from non-phenolic molecules of benzoic and cinnamic acid, respectively. The biosynthesis of the main phenolic acids present in mushrooms is described in **Figure 5**. Chemically, these compounds have at least one aromatic ring in which at least one hydrogen is substituted by a hydroxyl group. It would be interesting to highlight that *p*-hydroxybenzoic and *p*-coumaric acids present in Adlay bran (*Coix lacryma-jobi* L.) were reported as anti-inflammatory agents (Huang et al., 2014), but as far as we know, there are no reports on phenolic acids from mushrooms.


Entry
Starting Molecule
Enzyme
Final compound
(1)
Phenylalanine
Phenylalanine ammonia lyase
Cinnamic acid
(2)
Cinnamic acid
Oxidase (presumed ß-oxidation)
Benzoic acid
(3)
Benzoic acid
Benzoic acid 4-hydroxylase
p-Hydroxybenzoic acid
(4)
p-Hydroxybenzoic acid
p-Hydroxybenzoic acid 3-hydroxylase
Gentisic acid

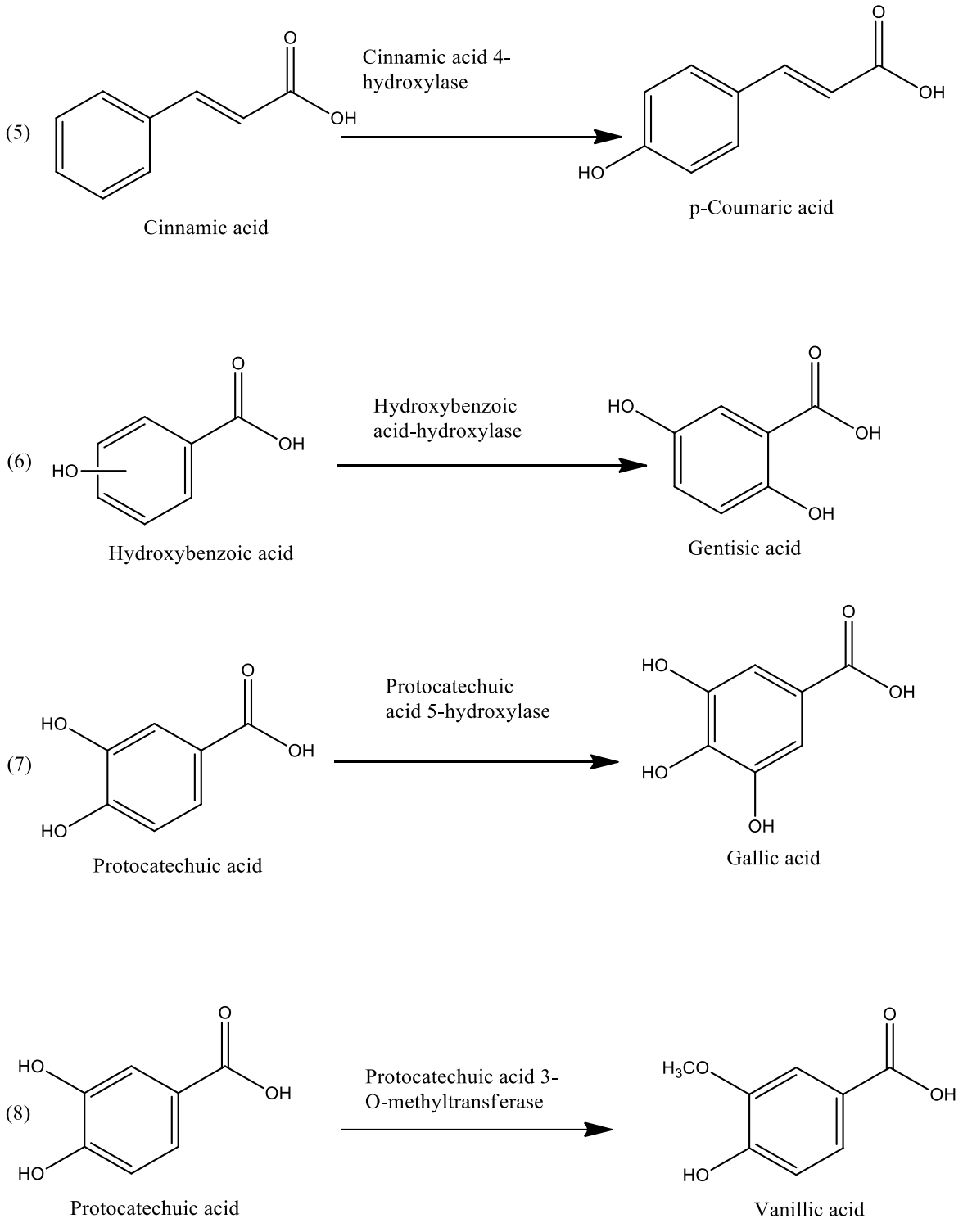
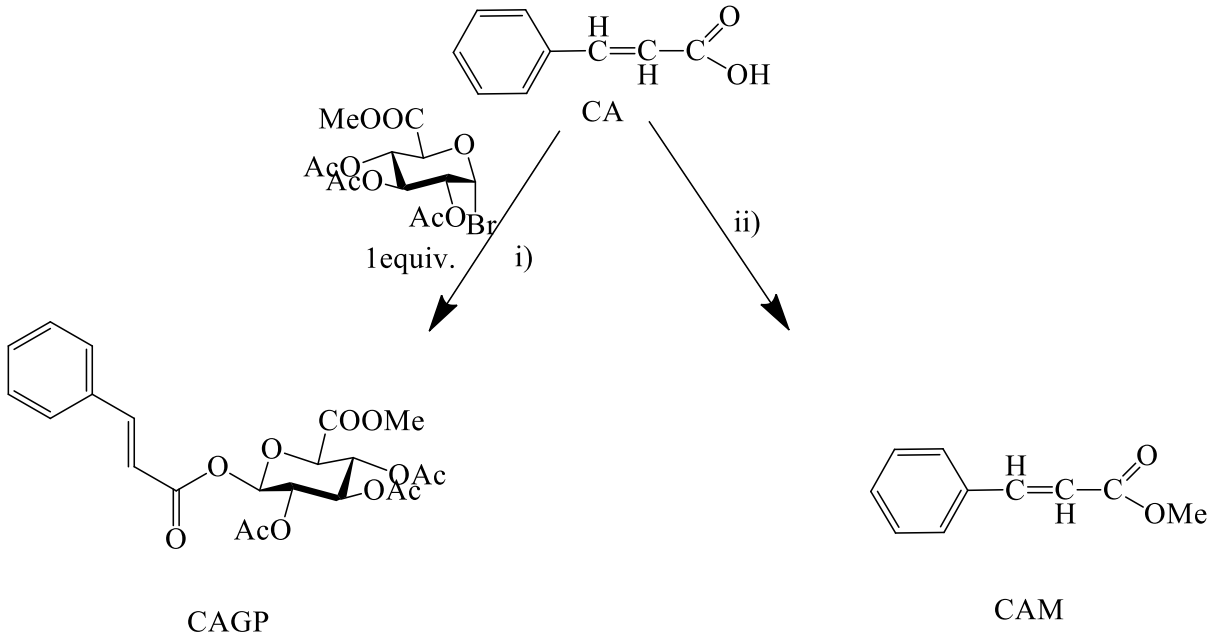


Figure 5. Main enzymes involved in the biosynthesis of phenolic acids through Shikimate pathway from L-phenylalanine.

There are only a few reports in the literature describing the synthesis of metabolites from phenolic acids to evaluate their bioactivities in comparison with the corresponding parent compounds. Piazzon et al. (2012), as well as our research group (Heleno et al., 2013a, 2014a), described the chemical or enzymatic synthesis of glucuronated, sulphated and methylated derivatives of several phenolic acids, obtaining structures similar to those formed *in vivo* after phenolic acid metabolism (**Figure 6-8**).



i) K_2CO_3 1.5 equivs, DMSO, Ar, RT, 24h;
ii) MeOH; H_2SO_4 , 5 days, R.T.

Figure 6. i) Glucuronidation of cinnamic acid (CA). CAGP- cinnamic acid glucuronide protected form, 2,3,4-tri-O-acetyl-1-cinnamoyl-D-glucuronic acid methyl ester (Heleno et al., 2013a); ii) Methylation of CA. CAM- methyl 3-phenylacrylate (Heleno et al., 2014a).

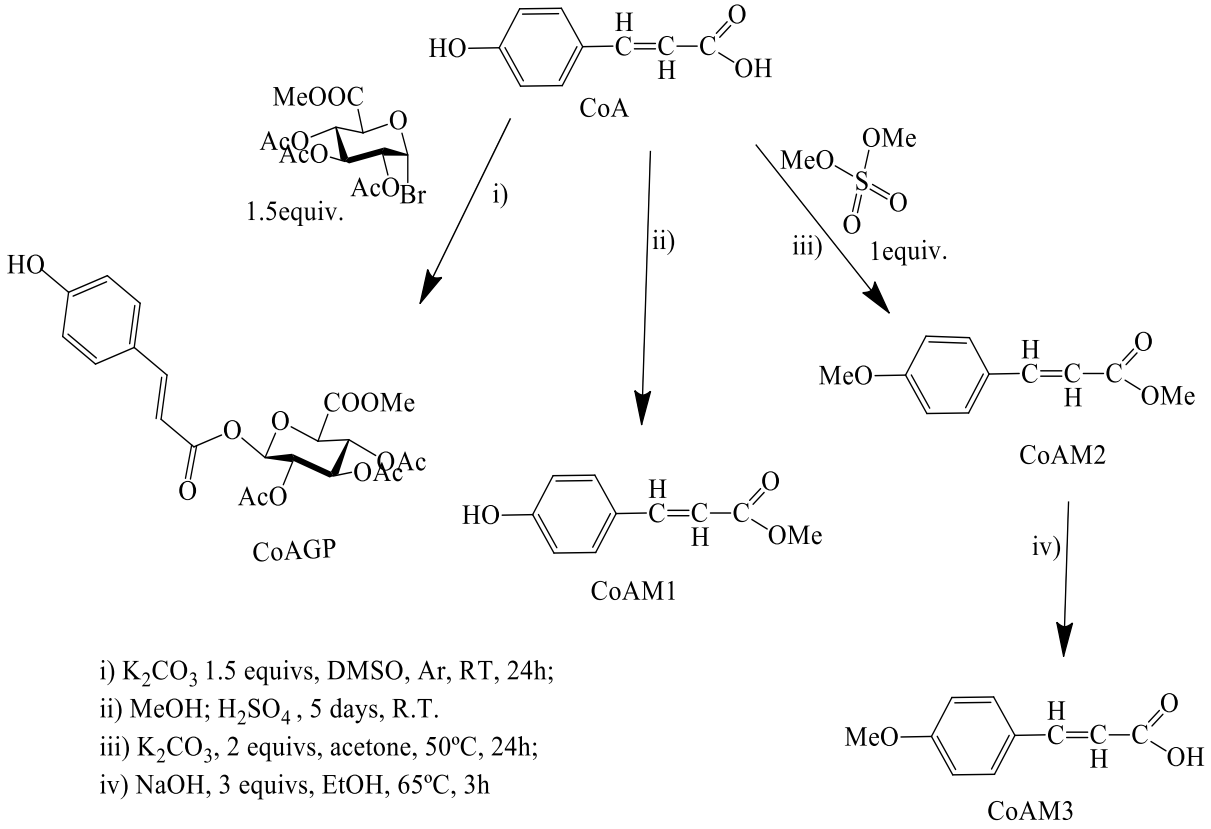


Figure 7. i) Glucuronidation of p-coumaric acid (CoA). CoAGP- p-Coumaric acid glucuronide protected form, 2,3,4-tri-O-acetyl-1-p-coumaroyl-D-glucuronic acid methyl ester (Heleno et al., 2014a) ii)-iv) Methylations of CoA. CoAM1- 3-(4-hydroxyphenyl) acrylate, CoA

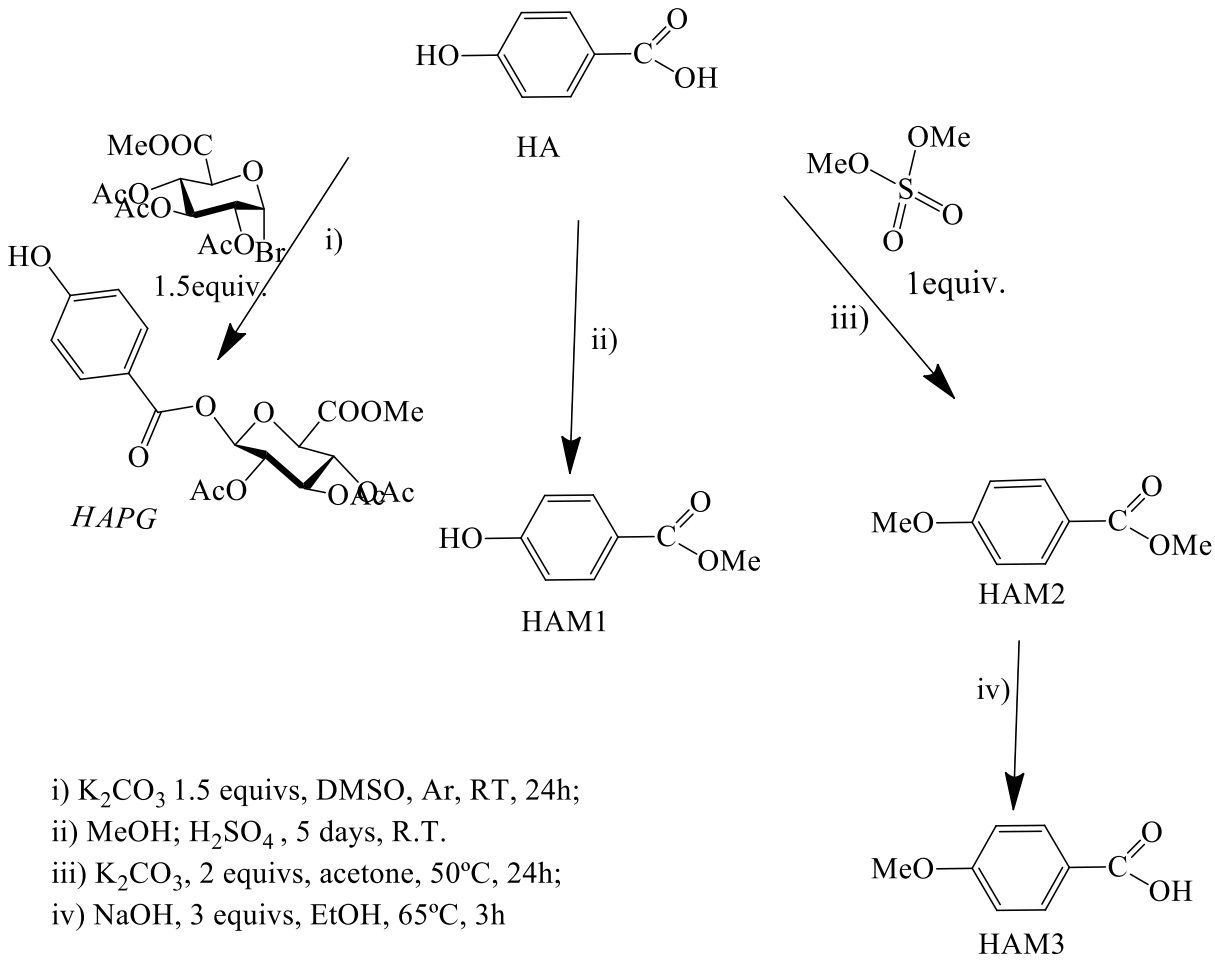


Figure 8. i) Glucuronidation of p-hydroxybenzoic acid (HA). HAGP- p-hydroxybenzoic acid protected form, 2,3,4-tri-O-acetyl-1-p-hydroxybenzoyl-D-glucuronic acid methyl ester (Heleno et al., 2013a). ii, iii, iv) Methylations of p-hydroxybenzoic acid, HAM1- methyl.

All the glucuronated, methylated and sulphated compounds mentioned above, and the respective parental phenolic acids, were studied for their bioactivities namely, antioxidant (Piazzon et al., 2012), antimicrobial (Heleno et al., 2013a, 2014a) and antitumor (Heleno et al., 2014a) properties, in order to compare the biological activities of the parental compounds before and after *in vivo* metabolism.

1.4. Objectives

We analyzed ethanolic extracts of ten wild mushroom species (*Amanita caesaria* (Scop.) Pers., *Boletus aereus* Bull., *B. edulis* Bull., *B. flagrans* Vittad., *B. impolitus* Fr., *B. reticulatus* Schaeff., *Cantharellus cibarius* Fr., *Lactarius deliciosus* (L. ex Fr.) S.F. Gray, *Macrolepiota procera* (Scop.) Singer and *Morchella esculenta* Fr.), collected in the Northeast of Portugal, and four cultivated species (*Agaricus bisporus* (J.E. Lange) Emil J. Imbach, *A. bisporus* Portobello (J.E. Lange) Emil J. Imbach, *Pleurotus eryngii* (DC.) Quél. and *Pleurotus. ostreatus* (Jacq. ex Fr.) P. Kumm.) were used in the present study for anti-inflammatory activity in LPS activated RAW 264.7 macrophages. Furthermore, after characterization of the extracts in terms of phenolic acids and related compounds, the identified individual parent molecules and their synthesised glucuronated and methylated derivatives were evaluated for the same bioactivity, in order to establish structure-activity relationships.

The specific objectives were:

1. To evaluate the anti-inflammatory activity of ethanolic extracts prepared from selected edible and wild mushrooms.
2. To chemically characterize the mushroom extract in terms of phenolic acids by HPLC-PDA (high performance liquid chromatography with photodiode array detection).
3. To evaluate the anti-inflammatory activity of the phenolic acids identified in the extracts, as also of their synthesized glucuronated and methylated derivatives.
4. Compare the activity of the extracts, individual phenolic acids and derivatives, in order to establish a structure-bioactivity relationship.

CHAPTER 2. EXPERIMENTAL SECTION

2.1 Materials and Samples

2.1.1. Mushroom samples

Ten wild mushroom species (*Amanita caesaria* (Scop.) Pers., *Boletus aereus* Bull., *B. edulis* Bull., *B. flagrans* Vittad., *B. impolitus* Fr., *B. reticulatus* Schaeff., *Cantharellus cibarius* Fr., *Lactarius deliciosus* (L. ex Fr.) S.F. Gray, *Macrolepiota procera* (Scop.) Singer and *Morchella esculenta* Fr.), collected in the Northeast of Portugal, and four cultivated species (*Agaricus bisporus* (J.E. Lange) Emil J. Imbach, *A. bisporus* Portobello (J.E. Lange) Emil J. Imbach, *Pleurotus eryngii* (DC.) Quél. and *Pleurotus. ostreatus* (Jacq. ex Fr.) P. Kumm.) (**Figure 9**) were used in the present study. All species were deposited in the herbarium of the School of Agriculture in Polytechnic Institute of Bragança, and have been previously characterized by the research group in terms of nutritional value and chemical composition (including primary and secondary metabolites) (Barros et al., 2009; Grangeia et al., 2011; Heleno et al., 2011; Reis et al., 2011; Pereira et al., 2012; Reis et al., 2012; Heleno et al., 2013b).

Their antioxidant, antimicrobial and antitumor properties were also previously evaluated by the group (http://esa.ipb.pt/biochemcore/index.php/studied-mushrooms).

Agaricus bisporus
(http://www.gbif.org/species)

Agaricus bisporus Portobello
(http://www.mycelia.be/en/strain)

Amanita caesaria
(http://www.cestaysetas.com)

Boletus aereus
(https://commons.wikimedia.org)

Boletus edulis
(http://www.mykoweb.com)

Boletus flagrans
(http://boletales.com)

Boletus impolitus
(http://monnuagedecreas.com)

Boletus reticulatus
(http://www.aranzadi.eus)

Cantharellus cibarius
(http://www2.muse.it)

Lactarius deliciosus
(https://www.flickr.com)

Macrolepiota procera
(http://www.aranzadi.eus)

Morchella esculenta
(https://commons.wikimedia.org)

Pleurotus eryngii
(http://www.boletsdesoca.com)

Pleurotus ostreatus
(http://www.mykoweb.com)

Figure 9. Studied mushroom species.

2.1.2. Reagents and solvents

Acetonitrile 99.9% was of high-performance liquid chromatography (HPLC) grade from Lab-Scan (Lisbon, Portugal). Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, Griess reagent system (Promega), DMSO, Glutamine, Non-essential amino acids, sulphorodamine B (SRB) and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich Co. (Saint Louis, MO, USA). All other chemicals and solvents were of analytical grade and purchased from common sources.

2.1.3. Equipment

The equipment used in the present work is listed in **Table 2**.

Table 2. List of equipment used in the present study.

Equipment	Company	Application
Digital balance	KERN ABS-N	Weight mushroom sample and reagents
Magnetic stirrer	ARE VELP Scientifica	Stirring the samples for proper extraction
Rotary evaporator	Heidolph (Hei-Vap)	Evaporation of the extraction solvent
Ultrasound bath	ELMA TRANSSONIC T 460/H	Proper dissolution of the extracts
HPLC-PDA	UFLC, Shimadzu Coperation, Kyoto, Japan	Characterization of the extracts in terms of phenolic acids
Air incubator	VWR INCU- Line	Preparation of culture media
CO_2 incubator	Heal force HF 151	Cells growth under controlled temperature, CO_2 and humidity
Inverted Microscope	Nikon ECLIPSE TS100	Analysis of the morphology of RAW 267.6 cells
Vertical laminar flow bench	Telstar Technologies Av/30/70	Safety manipulation of the animal cells
Centrifuge	HERMLE Z 206 A	Obtaining RAW 267.6 cell's suspensions
Microscope	Motic BA80 Series	Counting RAW 267.6 cells
Inverted Microscope	Nikon ECLIPSE TS100	Analysis of the morphology of RAW 267.6 cells
Microplates reader	ELX800 Biotek	Measuring the absorbance at 540nm

2.2. Methodology

2.2.1. Extracts preparation

Dried mushroom powder of each species (0.5 g) was extracted with ethanol (15 mL), by maceration with stirring for 1 h. Then, the extract was filtered through Whatman no 4 filter paper and the extraction procedure was repeated one more time. The filtrate was rotary evaporated to remove ethanol (**Figure 10**) and the extraction yield was calculated by measuring the extract weight in relation to the initial mass sample (**Table 3**).

Figure 10. Extract solutions and ethanol removal.

Table 3. Extraction yields obtained for each mushroom species.

Mushroom species	Final mass of extract (mg)	Extraction yield (%)
Agaricus bisporus	29.0 ± 0.6	5.80
Agaricus bisporus portobelo	22.3 ± 0.4	4.50
Amanita caesaria	23.8 ± 0.4	4.76
Boletus aereus	40.9 ± 0.8	8.18
Boletus edulis	36.0 ± 0.8	7.20
Boletus flagrans	25.4 ± 0.6	5.08
Boletus impolitus	24.0 ± 0.7	4.80
Boletus reticulatus	43.2 ± 0.9	8.64
Cantharellus cibarius	21.2 ± 0.6	4.24
Lactarius deliciosus	23.8 ± 0.4	4.76
Macrolepiota procera	26.8 ± 0.5	5.36
Morchella esculenta	22.0 ± 0.4	4.44
Pleurotus eryngii	49.0 ± 0.8	9.80
Pleurotus ostraetus	17.2 ± 0.3	3.44

2.2.2. Chemical characterization of the extracts

The dry mass of each mushroom extract was re-dissolved in water/ethanol (50:50, *v/v*) (**Figure 11**) and filtered through a 0.22 µm nylon disposable filter for HPLC analysis. The analysis was performed using a Shimadzu 20A series ultra-fast liquid chromatograph (UFLC, Shimadzu Coperation, Kyoto, Japan). Separation was achieved on a Waters Spherisorb S3 ODS2 C_{18} column (3 µm, 150 mm x 4.6 mm) column thermostatted at 35ºC. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was 10% B to 15% B over 5 min, 15–25% B over 5 min, 25–35% B over 10 min, isocratic 50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Detection was carried out in a photodiode array detector (PDA), using 280 nm as the preferred wavelength.

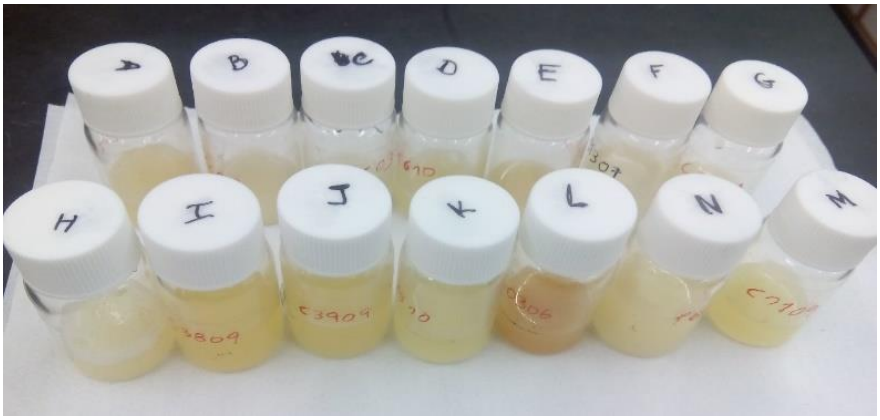


Figure 11. Extract solutions ready to be filtered for HPLC analysis.

The phenolic acids (group of phenolic compounds identified in the samples) were quantified by comparison of the area of their peaks recorded at 280 nm with calibration curves obtained from commercial standards of each compound: *p*-hydroxybenzoic acid (y = 113523x, R^2=0.999), *p*-coumaric acid (y = 433521x, R^2=0.998) and cinnamic acid (y = 583527x, R^2=0.998), 5 to 80 μg/mL. The results were expressed as milligrams per mL of extract.

2.2.3. Phenolic acids and synthesised derivatives

p-Hydroxybenzoic acid, *p*-coumaric acid and cinnamic acid, identified in the mushroom extracts, were purchased from Sigma-Aldrich Co. (Saint Louis, MO, USA). Methylated and glucuronated derivatives of the identified compounds (**Figure 6,7 and 8**) were previously synthesized and completely characterized by the authors (Heleno et al., 2013a; Heleno et al., 2014).

2.2.4. Evaluation of the anti-inflammatory activity

2.2.4.1. Preparation of cells growth medium.

DMEM was supplemented with 10% heat-inactivated FBS, 1% penicillin and streptomycin, 1% glutamine and 1% non-essential amino acid. This preparation procedure was carried out in the vertical laminar flow bench in order to offer optimum protection against contaminations. It was then stored in an incubator with natural air circulation.

2.2.4.2 Cells treatment.

For the anti-inflammatory activity assay, the ethanolic extracts were dissolved in water at 8 mg/mL, while the identified individual compounds and their synthesised methylated and

glucuronated derivatives were dissolved in DMSO at 50% concentration in stock solution. For the different assays, the solutions were then submitted to further dilutions (400 µg/mL to 50 µg/mL and 2500 µM to 39 µM for the extracts and compounds, respectively) in order to determine effective concentrations.

The mouse macrophage-like cell line RAW 264.7 was cultured in DMEM medium supplemented with 10% heat-inactivated FBS, glutamine and antibiotics at 37 °C under 5% CO_2, in humidified air.

For each experiment, cells were detached with a cell scraper. A cell density of $5x10^5$ cells/mL was used, and the proportion of dead cells was less than 5%, according to Trypan blue dye exclusion test.

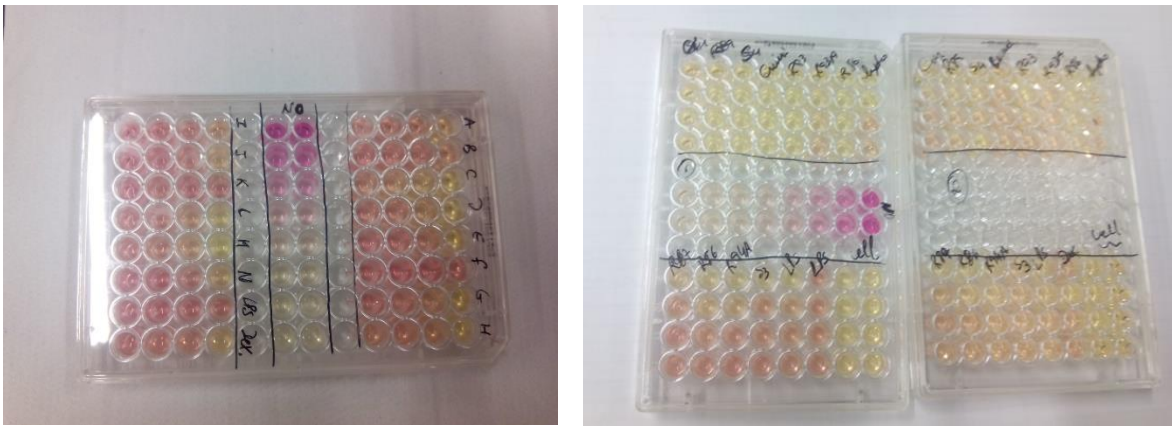
Cells were seeded in 96-well plates at 150,000 cells/well and allowed do attach to the plate overnight. Then, cells were treated with the different concentrations of each one of the extracts for 1h. Dexamethasone (50 µM) was used as a positive control for the experiment. The following step was the stimulation with LPS (1 µg/mL) for 18h. The effect of all the tested samples in the absence of LPS was also evaluated, in order to observe if they induced changes in nitric oxide (NO) basal levels. In negative controls, no LPS was added. Both extracts and LPS were dissolved in supplemented DMEM.

2.2.4.3. Nitric oxide determination.

For the determination of nitric oxide, Griess Reagent System kit was used, which contained sulphanilamide, N-(1-napthyl) ethylenediamine hydrochloride (NED) and nitrite solutions. A reference curve of the nitrite (sodium nitrite 100 µM to 1.6 µM; y=0.0064x+0.1311, R^2=0.9981) was prepared in a 96-well plate. The cell culture supernatant (100 µL) was transferred to the plate and mixed with Sulphanilamide and NED solutions, 5-10 minutes each, at room temperature.

The nitric oxide produced was determined by measuring the absorbance at 540 nm (microplate reader) (**Figure 12**), and by comparison with the standard calibration curve. The percentage of inhibition of the NO production was calculated, for each sample concentration, by the equation: [(Substrate concentration - Basal cells)/LPS – Basal cells] x100

For an easier comparison of the results, EC_{50} values were calculated based on 50% of inhibition of NO production.



A) **B)**

Figure 12. Microplates showing the NO assay with A) ethanolic mushroom extract and B) phenolic acids, already stimulated with 1 µg/mL LPS and Griess Reagent kit to be read at 540 nm.

2.2.5. Statistical analysis

For all the experiments three samples were analyzed and all the assays were carried out in triplicate. The results are expressed as mean values ± standard deviation (SD). The differences between the different samples were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with $\alpha = 0.05$, coupled with Welch's statistic. This treatment was carried out using SPSS v. 22.0 program.

CHAPTER 3. RESULTS AND DISCUSSION

Ethanolic extracts were prepared from ten wild mushroom species *Amanita caesaria, Boletus aereus, B. edulis, B. flagrans, B. impolitus, B. reticulatus, Cantharellus cibarius, Lactarius deliciosus, Macrolepiota procera, Morchella esculenta*, and four cultivated species *Agaricus bisporus, A. bisporus* Portobello, *Pleurotus eryngii.* and *Pleurotus. ostreatus*. The compounds identified in the extracts by HPLC-PDA are presented in **Table 4**.

Table 4. Compounds identified in the mushroom ethanolic extracts by HPLC-PDA.

Mushroom species	**Extraction Yield (%)**	***p*-Hydroxybenzoic acid (µg/g)**	***p*-Coumaric acid (µg/g)**	**Cinnamic acid (µg/g)**
Agaricus bisporus	6.9 ± 0.7^{abcd}	nd	nd	149 ± 2^{d}
Agaricus bisporus portobelo	6.0 ± 0.1^{bcd}	nd	nd	11 ± 1^{hi}
Amanita caesaria	5.4 ± 0.5^{cd}	57 ± 3^{e}	nd	156 ± 3^{d}
Boletus aereus	7.7 ± 0.6^{abc}	43 ± 1^{f}	74 ± 1^{b}	50 ± 3^{f}
Boletus edulis	10.4 ± 0.5^{a}	nd	nd	14.2 ± 0.4^{gh}
Boletus flagrans	4.9 ± 0.3^{cd}	nd	nd	6.1 ± 0.3^{i}
Boletus impolitus	4.1 ± 0.5^{d}	125 ± 9^{c}	45 ± 2^{c}	505 ± 12^{c}
Boletus reticulatus	9.5 ± 0.3^{ab}	nd	nd	20.3 ± 0.1^{g}
Cantharellus cibarius	4.0 ± 0.3^{d}	151 ± 2^{b}	nd	71 ± 1^{e}
Lactarius deliciosus	3.7 ± 0.4^{d}	108 ± 5^{d}	nd	67 ± 1^{e}
Morchella esculenta	4.0 ± 0.8^{d}	nd	nd	71 ± 3^{e}
Macrolepiota procera	3.7 ± 0.4^{d}	nd	nd	522 ± 1^{b}
Pleurotus eryngii	10.1 ± 0.6^{a}	nd	nd	16 ± 1^{gh}
Pleurotus ostraetus	4.0 ± 0.6^{d}	297 ± 5^{a}	171 ± 1^{a}	619 ± 3^{a}

nd- not detected. ANOVA In each column, different letters mean statistical significant differences ($p<0.05$) between samples.

NO is a signalling molecule that plays critical role in the pathogenesis of inflammation (Sharma et al., 2007). Although physiological levels of NO are required for events such as vasodilation, angiogenesis, and neurotransmission, the overproduction of NO under pathological conditions can be a toxic and pro-inflammatory mediator that induces inflammation (Guzik et al., 2003). Therefore, the inhibition of NO production under

inflammatory stimuli is relevant to the development of anti-inflammatory agents (Biesalski, 2007). The ability of ethanolic extracts of the fourteen different mushroom species to modulate the production of inflammatory mediator NO was evaluated in RAW 264.7 macrophages by pre-treating cells with various concentrations of the mushroom extract before stimulation with 1 µg/mL LPS.

LPS is a cell wall component of Gram-negative bacteria, and plays a central role in the pathogenesis of several diseases (Sachithanandan et al., 2011). When macrophages are exposed to LPS, the LPS binds to receptors that activate several signalling pathway that intern result in activation of NF-κB. When the NF-κB signalling pathway is activated, macrophages secrete NO and pro-inflammatory cytokines. Hence, natural agents that regulate the production of various cytokines and suppress the overproduction of NO may have protective roles in inflammation-related diseases (Nam, 2006).

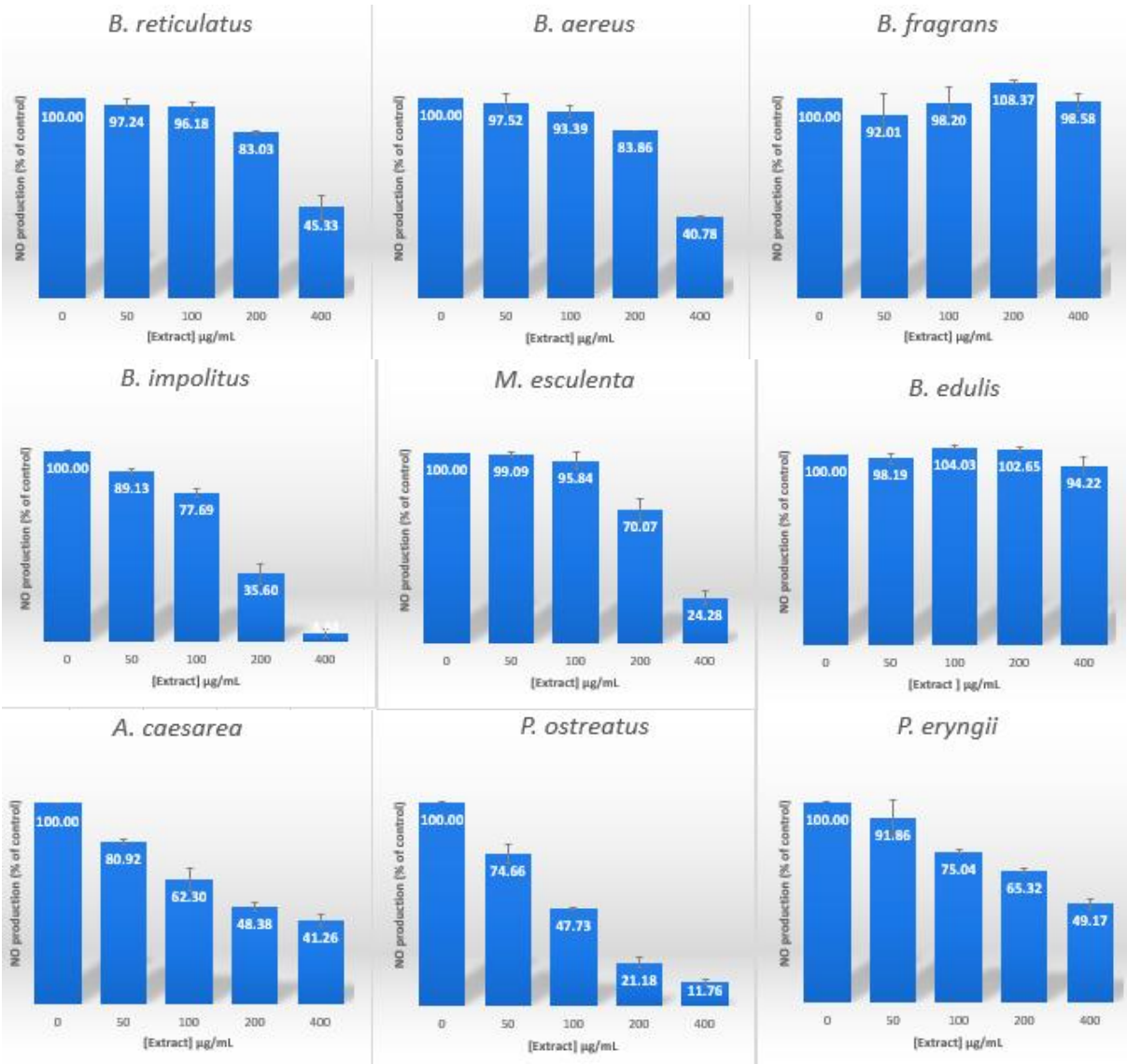
The effects of the ethanolic extracts on the production of inflammatory mediators (NO) in RAW 264.7 macrophages, upon stimulation with LPS, are shown in **Figure 13.** The results indicate that some extracts inhibited LPS-induced NO production in a dose-dependent manner. For an easier comparison of the results, EC_{50} values were calculated based on 50% of inhibition of NO production (**Table 5**).

Table 5. EC_{50} values responsible for 50% of reduction of NO production in RAW 264.7 cell line.

Mushroom species	EC_{50} values (µg/mL)	Mushroom species	EC_{50} values (µg/mL)
Agaricus bisporus	190 ± 6^{ef}	*Boletus reticulatus*	378 ± 28^{ab}
Agaricus bisporus portobelo	$>400^{a}$	*Cantharellus cibarius*	202 ± 17^{e}
Amanita caesaria	186 ± 7^{ef}	*Lactarius deliciosus*	253 ± 14^{d}
Boletus aereus	357 ± 3^{b}	*Macrolepiota procera*	162 ± 2^{g}
Boletus edulis	$>400^{a}$	*Morchella esculenta*	287 ± 9^{c}
Boletus flagrans	$>400^{a}$	*Pleurotus eryngii*	388 ± 17^{a}
Boletus impolitus	166 ± 10^{fg}	*Pleurotus ostraetus*	96 ± 1^{h}

EC_{50} values correspond to 50% of inhibition of the NO production in comparison with the negative control (100% of NO production). In the columns, different letters mean statistical significant differences ($p<0.05$) between samples. Dexamethaxone EC_{50} value = 16 ± 2 µg/mL.

The most efficient species was *P. ostreatus* (96 ± 1 µg/mL), followed by *M. procera* (162 ± 2 µg/mL), *B. impolitus* (166 ± 10 µg/mL) and *A. bisporus* (190 ± 6 µg/mL). These results are in agreement with the reports of Moro et al. (2012) and Gunawardena et al. (2014) that described anti-inflammatory activity, by decreasing NO levels in RAW 264.7 cells, of ethanolic and methanolic extracts of *A. bisporus*, *C. cibarius*, *L. deliciosus* and *P. ostreatus* (**Table 5**). The ethanolic extracts from *A. bisporus* Portobello, *B. edulis* and *B. flagrans* appeared as the less active, showing EC_{50} values above 400 µg/mL. Moro et al. (2012) reported some activity in the case of methanolic extracts of *B. edulis* with 10% inhibition of NO production at concentrations of 500 µg/mL.



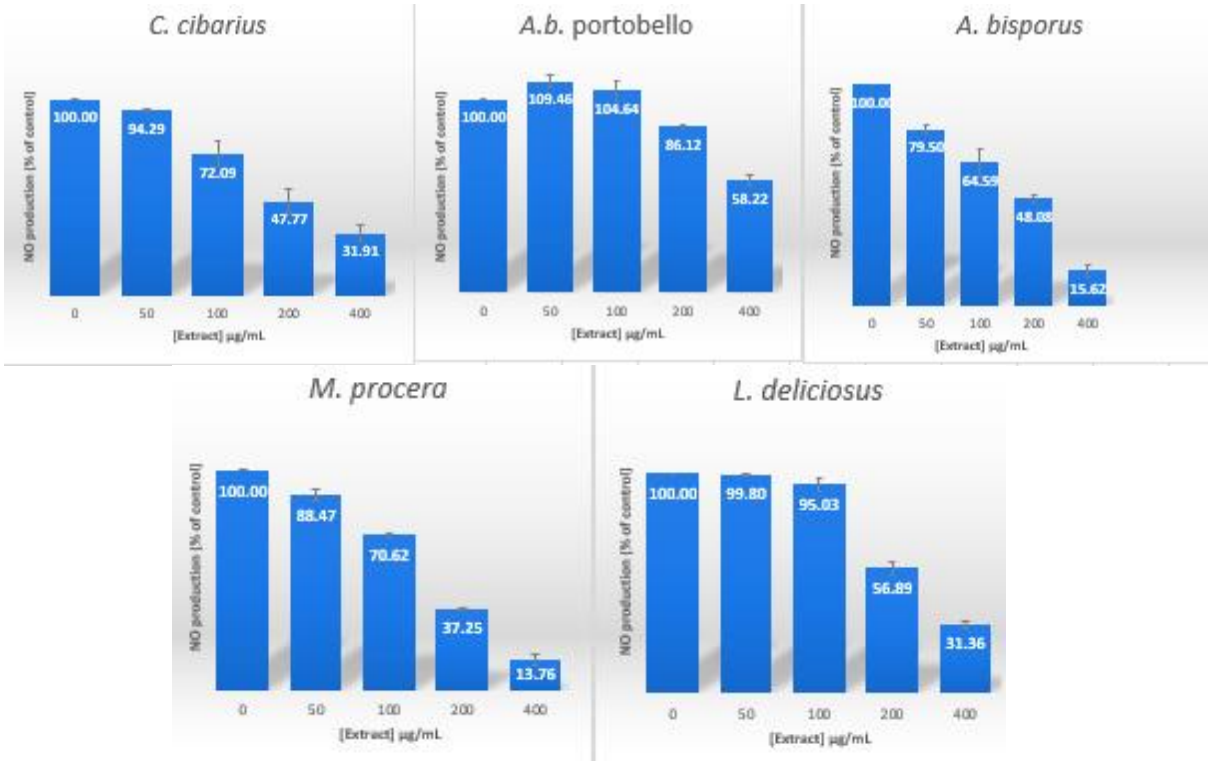


Figure 13. . Effects of the ethanolic mushroom extracts showing the % NO produced by RAW 264.7 cell line. Mean values and standard errors, expressed in relation to the negative control (without extract) - 100% of production.

Although many substances may participate in the anti-inflammatory activity, phenolic compounds have been largely recognised as natural molecules with anti-inflammatory effects. Positive correlations have been found between phenolic compounds and anti-inflammatory effects (Cheung, Cheung, & Ooi, 2003; Kim et al., 2008). In the present study, it was also observed that the extract with the highest anti-inflammatory activity showed the highest levels of cinnamic and phenolic acids.

To demonstrate this supposition, the activity of the individual compounds present in the extracts were further evaluated (**Table 4**). Cinnamic acid (CA) showed the highest anti-inflammatory activity presenting the lowest EC_{50} values (182 ± 16 µM), followed by *p*-hydroxybenzoic (239 ± 29 µM) and *p*-coumaric (442 ± 33 µM) acids. These results are in agreement with the ones reported by our research group for the antimicrobial activity of these

compounds, where cinnamic acid also revealed the strongest activity followed by *p*-hydroxybenzoic and *p*-coumaric acids (Heleno et al., 2014a).

As phenolic acids are metabolized in the organism and suffer conjugation reactions originating different metabolites such as glucuronated and methylated derivatives, a change in their effects or activity may also occur. Thus, the bioactivity of the parent molecule can be increased, decreased or maintained (Heleno et al., 2015). Hereby the glucuronated and methylated derivatives of the considered acids were analysed and compared to the one of the parent molecule (**Figure 14**). Among the glucuronated derivatives, CoA-GP (glucuronated derivative of *p*-coumaric acid) presented strong anti-inflammatory activity (58 ± 5 µM), being comparable to the activity of the standard dexamethaxone (40 ± 4 µM), followed by the glucuronated derivatives of cinnamic (CA-GP) (179 ± 71 µM) and *p*-hydroxybenzoic (HA-GP) (1901 ± 104 µM) acids. Regarding the methylated derivatives, those of *p*-coumaric acid presented higher activity than the ones of cinnamic or *p*-hydroxybenzoic acids; in particular, the methylated derivative CoA-M1, with an ester instead of the carboxylic group, revealed very strong activity (35 ± 2 µM), very close to the dexamethaxone value (40 ± 4 µM).

Comparing the activity of each parent molecule and the corresponding glucuronated and methylated derivatives, the order was as follows: *p*-hydroxybenzoic acid: HA > HA-M3 > HA-M2 >HA-M1 > HA-GP; *p*-coumaric acid: CoA-M1 > CoA-GP > CoA-M2 > CoA-M3 > CoA; and cinnamic acid: CA-GP > CA > CA-M. *p*-Hydroxybenzoic acid showed higher activity than the corresponding derivatives, with HA-M3 as the most active compound; the decreased anti-inflammatory activity observed for HA-M1, HA-M2 and HA-GP could be explained by the esterification of the carboxylic group. On the contrary, all *p*-coumaric acid derivatives showed higher activity than the parent molecule (CoA), particularly the methylated compound CoA-M1 and CoA-GP, suggesting that in this case the esterification of the carboxylic group together with the free OH group in *para* position could be relevant features for their anti-inflammatory activity. The glucuronated derivative of cinnamic acid (CA-GP) maintained its activity in comparison with the parent molecule (CA), while the methylated one (CA-M) decreased it.

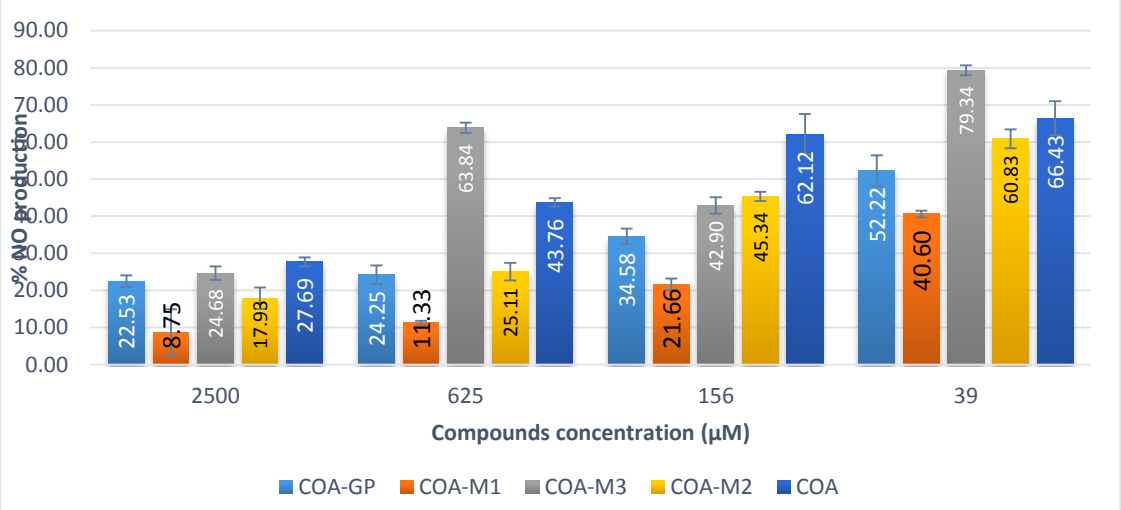
These results are in relatively good agreement with the ones previously reported by our research group comparing the antimicrobial activity of *p*-hydroxybenzoic, *p*-coumaric and cinnamic acids and their respective methylated and glucuronated derivatives. The

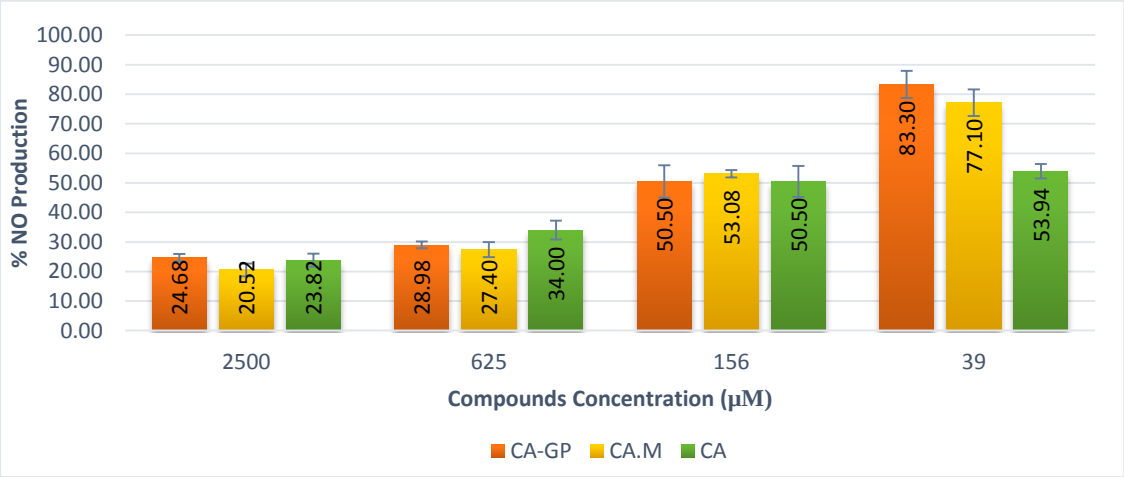
methylation of *p*-hydroxybenzoic and cinnamic acids decreased the antimicrobial activity, while the methylation of *p*-coumaric acid increased it (Heleno et al., 2014a); moreover, the glucuronidation of the parent molecules also decreased the antimicrobial activity, except for HA-GP that showed higher antifungal activity against some pathogenic strains (Heleno et al., 2013a).

Table 6. EC50 values responsible for 50% of reduction of NO production in RAW 264.7 cell line

***p*-Hydroxybenzoic acid and derivatives**		***p*-Coumaric acid and derivatives**		**Cinnamic acid and derivatives**	
HA	239 ± 29^{c}	CoA	442 ± 33^{a}	CA	182 ± 16^{b}
HA-GP	1901 ± 104^{a}	CoA-GP	58 ± 5^{c}	CA-GP	179 ± 17^{b}
HA-M1	1825 ± 120^{a}	CoA-M1	35 ± 2^{c}	CA-M	224 ± 16^{a}
HA-M2	526 ± 26^{b}	CoA-M2	128 ± 10^{b}		
HA-M3	509 ± 47^{b}	CoA-M3	129 ± 6^{b}		

EC_{50} values correspond to 50% of inhibition of the NO production in comparison with the negative control (100% of NO production). In each column, different letters mean statistical significant differences ($p<0.05$) between compounds. Dexamethaxone EC_{50} value = 40 ± 4 μM.





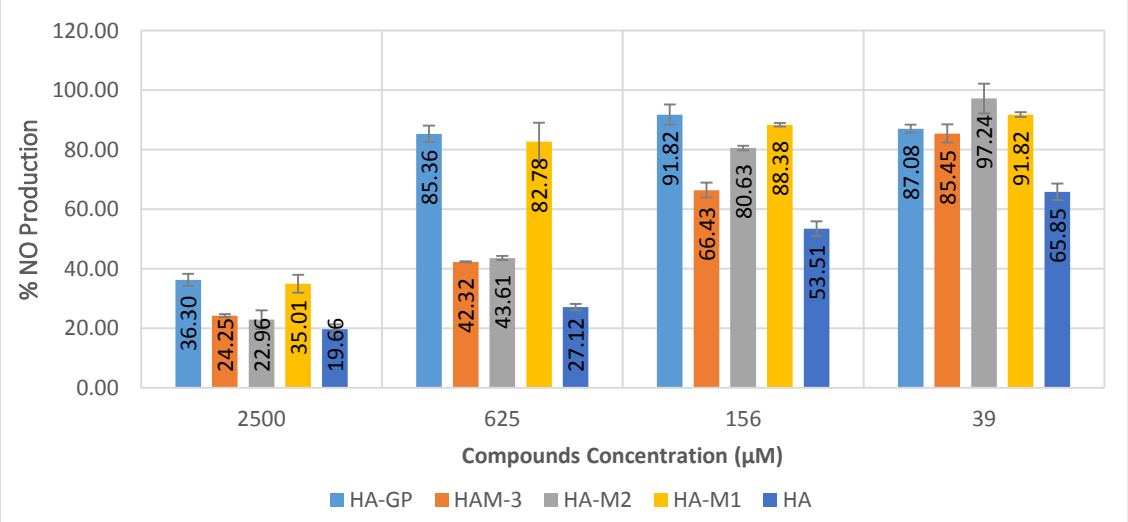


Figure 14. Effects of the synthesised derivatives and parent molecules showing the % NO produced by RAW 264.7 cell line. Mean values and standard errors, expressed in relation to the negative control (without compound) - 100% of production.

CHAPTER 4. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In this study, we investigated the anti-inflammatory effects of ethanolic extracts from ten wild mushroom species and four cultivated species using the LPS-stimulated macrophage cell RAW264.7 as a model system. Although we observed variations in the anti-inflammatory activity among mushroom species, the results clearly indicate that the most efficient species were *P. ostreatus* (96 ± 1 µg/mL), followed by *M. procera* (162 ± 2 µg/mL), *B. impolitus* (166 ± 10 µg/mL) and *A. bisporus* (190 ± 6 µg/mL). They all had an inhibitory effect on the production of the pro-inflammatory mediator NO due to the presence of important bioactive compounds. Therefore, each ethanolic extract was chemically characterized in terms of phenolic acids and the extracts were found to contain several active molecules (*p*-hydroxybenzoic acid, *p*-coumaric acid and cinnamic acid), which have been suggested to play an important role in controlling inflammatory diseases.

Furthermore, the individual phenolic acids present in the extracts and their synthesized derivatives were further evaluated in the same model system, LPS-stimulated macrophage cells RAW264.7. Cinnamic acid (CA) showed the highest anti-inflammatory activity presenting the lowest EC_{50} values (182 ± 16 µM), followed by *p*-hydroxybenzoic (239 ± 29 µM) and *p*-coumaric (442 ± 33 µM) acids. Comparing the activity of each parent molecule and the corresponding glucuronated and methylated derivatives, the order was as follows: *p*-hydroxybenzoic acid: HA > HA-M3 > HA-M2 >HA-M1 > HA-GP; *p*-coumaric acid: CoA-M1 > CoA-GP > CoA-M2 > CoA-M3 > CoA; and cinnamic acid: CA-GP > CA > CA-M. These results suggest that the phenolic acids present in the ethanolic mushroom extracts may contain some useful preventive and therapeutic agents against inflammatory responses.

Overall, the mushroom species: *P. ostreatus, M. procera, B. impolitus* and *A. bisporus* revealed the strongest anti-inflammatory potential presenting the highest inhibition of NO production. These mushroom species also revealed the highest concentration in cinnamic acid, which was also the individual compound that presented the strongest anti-inflammatory activity and, therefore, could play an important role in the observed activity. However, the conjugation reactions occurring in the organism can change the chemical structure of cinnamic and phenolic acids increasing or decreasing their *in vivo* anti-inflammatory activity. The possible metabolites previously synthesised by the authors and tested in the present work

are still displaying activity, in some cases like CoA-GP and CoA-M1 higher than the parent compound and very close to the activity exhibited by dexamethaxone used as anti-inflammatory standard.

This research work has demonstrated the importance of mushrooms as potential bio-factories for the production of natural anti-inflammatory compounds. The bioactivities of these compounds are exhibited through the downregulation of different types of inflammatory mediators. In addition to the high potential application of anti-inflammatory molecules from mushrooms in medical applications, they can also be used in cosmeceutical products as natural active ingredients.

However, the future medical application of anti-inflammatory compounds isolated from mushrooms faces several challenges. Firstly, most of the studied mushrooms are not cultivable in greenhouses, and thus their availability is both seasonal and highly affected by climatic changes. Secondly, the contents of the bioactive ingredients vary widely between samples, dependent on the collection time and procedure, the season, the environment and the genotype. Thirdly, mushroom cultivation needs to be run according to the current Good Manufacturing Practice (cGMP) requirements for the production of bioactive medicinal compounds. Fourthly, there is a lack of validated standard testing protocols to guarantee the quality and the efficacy of mushroom products for pharmaceutical applications.

Hence, more research should be done on the development of mushroom cultivation and extraction processes under fully sterile conditions so as to obtain bioactive metabolites for pharmaceutical applications. Also, further experiments are needed to be able to elucidate the anti-inflammatory mechanism of action of these natural biological agents.

The results presented in this work were published in Food Research International, a journal from Elsevier:

Oludemi Taofiq, Ricardo C. Calhelha, Sandrina Heleno, Lillian Barros, Anabela Martins, Celestino Santos-Buelga, Maria João R.P. Queiroz, Isabel C.F.R. Ferreira. **The contribution of phenolic acids to the anti-inflammatory activity of mushrooms: screening in phenolic extracts, individual parent molecules and synthesized glucuronated and methylated derivatives.** Accepted for publication in Food Research International (24/07/2015).

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